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# IRS2 and PTEN are key molecules in controlling insulin sensitivity in podocytes



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## ABSTRACT

Insulin signaling to the glomerular podocyte is important for normal kidney function and is implicated in the pathogenesis of diabetic nephropathy (DN). This study determined the role of the insulin receptor substrate 2 (IRS2) in this system. Conditionally immortalized murine podocytes were generated from wild-type (WT) and insulin receptor substrate 2-deficient mice (*Irs2*<sup>−/−</sup>). Insulin signaling, glucose transport, cellular motility and cytoskeleton rearrangement were then analyzed. Within the glomerulus IRS2 is enriched in the podocyte and is preferentially phosphorylated by insulin in comparison to IRS1. *Irs2*<sup>−/−</sup> podocytes are significantly insulin resistant in respect to AKT signaling, insulin-stimulated GLUT4-mediated glucose uptake, filamentous actin (F-actin) cytoskeleton remodeling and cell motility. Mechanistically, we discovered that *Irs2* deficiency causes insulin resistance through up-regulation of the phosphatase and tensin homolog (PTEN). Importantly, suppressing PTEN in *Irs2*<sup>−/−</sup> podocytes rescued insulin sensitivity. In conclusion, this study has identified for the first time IRS2 as a critical molecule for sensitizing the podocyte to insulin actions through its ability to modulate PTEN expression. This finding reveals two potential molecular targets in the podocyte for modulating insulin sensitivity and treating DN.

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## 1. Introduction

Diabetic nephropathy (DN) is a major complication of diabetes, with up to 40% of patients experiencing this problem [1]. It is the leading cause of end-stage renal failure in the world [2]. The natural history of DN is dominated by progressive albuminuria [3], making the glomerular podocyte an attractive early target cell. Podocytes are highly specialized, terminally differentiated, cells that are critically important for preventing albumin to escape into the urine. Indeed there are now in excess of 35 podocyte specific human mutations, all of which result in albuminuria [4]. Albuminuria is detrimental as it is a prelude to end-stage renal

failure in diabetic and non-diabetic diseases [2]. It is also independently linked to cardiovascular morbidity and mortality [3]. Podocytes are important early target cells in the pathogenesis of DN as evidenced by podocyte number and morphology being excellent predictors of DN progression [5–10]. Previous studies by our group [11,12] and others [13,14] have shown that podocytes are insulin responsive cells, and that they signal through the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways to induce a variety of biological effects. Moreover, a loss of podocyte insulin sensitivity in the intact perfused glomerulus results in an albuminuric phenotype with features reminiscent of DN but in a normoglycaemic environment [12,15]. Recently, it has been shown that insulin signaling is also important in modulating other cellular processes in the podocyte including endoplasmic reticulum stress [14] and mitochondrial function [16]. Therefore, understanding the key signaling pathways and proteins that control insulin actions in this cell type is highly desirable.

A critical node in the insulin signaling pathway is the insulin receptor substrate (IRS) family of proteins. The IRS family contains at least four members, including IRS1, IRS2, IRS3, and IRS4. IRS1 and IRS2 are

Abbreviations: F-actin, filamentous actin; IR, insulin receptor; PTEN, phosphatase and tensin homolog.

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the main mediators of insulin signaling in peripheral tissues. IRS3 is predominantly expressed in mouse adipose tissue and IRS4 is expressed in the pituitary, thymus and brain [17]. IRS1 and IRS2 are docking proteins that transduce signaling cascades emerging from the IR that regulate a variety of processes including metabolism, cellular growth, development and survival [18]. Although IRS1 and IRS2 share similar protein structure and expression patterns, several lines of evidence suggest a tissue-specificity of IRS-mediated signaling in growth and metabolism [19–21]. Mice with whole body deletion of IRS1 (*Irs1*<sup>−/−</sup>) display reduced body size, insulin resistance and  $\beta$ -cell hyperplasia [19]. In contrast, whole body IRS2-deficient mice (*Irs2*<sup>−/−</sup>) have defects in hepatic insulin action [20–22] and  $\beta$ -cell insufficiency leading to hyperglycemia due to impaired insulin-like growth factor-1 (IGF-1) receptor mitogenic signaling [20]. Consequently, male *Irs2*<sup>−/−</sup> mice develop type-2-like diabetes and most die around 16 weeks of age due to diabetic complications. Conversely, IRS3 and IRS4 are not essential for normal growth, glucose homeostasis or glucose transport, as illustrated by whole body murine knockout models [17].

This study shows that within the glomerulus IRS2 is enriched in the podocyte and that its deletion causes a loss of biochemical and functional metabolic insulin signaling in these cells. Furthermore, IRS2 loss in the podocyte up-regulates the phosphatase and tensin homolog (PTEN) which inhibits PI3K signaling. Importantly, insulin signaling can be rescued in IRS2-deficient podocytes by suppressing PTEN expression.

## 2. Materials and methods

Additional methods are included in the supplementary material.

### 2.1. Generation of immortalized podocyte cell lines

Conditionally immortalized murine podocytes were generated from wild-type (mPodWT) and *Irs2*<sup>−/−</sup> (mPodIRS2KO) mice on a mixed C57BL6 x 129Sv genetic background at 12 weeks of age following the protocol previously described [23]. Different clones of each cell line were studied to avoid possible clonal effects

### 2.2. Cellular stimulation

To assess insulin signaling, differentiated mPodWT and mPodIRS2KO were starved of fetal bovine serum (FBS) and insulin for at least 8 h prior to stimulation. Cells were then treated with insulin or IGF-1 at doses ranging from 1 to 100 nM for 15 min. When insulin doses are not specified, 100 nM was used. To assess post-puromycin stress, differentiated mPodWT and mPodIRS2KO were deprived of FBS for at least 8 h prior to stimulation with puromycin aminonucleoside (50  $\mu$ g/ml), a podocyte toxin for 18 h

### 2.3. Measurement of 2-deoxyglucose uptake

For the analysis of insulin-induced 2-deoxyglucose (2-DOG) uptake, we followed the protocol previously described [12]. Briefly, podocytes were grown to confluence and each experiment was performed with equal matched samples of basal and insulin-stimulated wells. After 12 h of FBS and insulin starvation, cells were stimulated for 15 min with insulin and matched control wells treated with PBS (vehicle). [<sup>3</sup>H] 2-deoxyglucose (DOG) was added to the basal and insulin-stimulated cells at a concentration of 50  $\mu$ M (1  $\mu$ Ci/ml) for the last 5 min of the incubation

### 2.4. Subcellular fractionation

Cells were homogenized in lysis buffer as previously described [24]. After sequential centrifugations, the Triton X-100-soluble component (plasma membrane fraction) was separated from the Triton-insoluble

material (cytoskeletal fraction) by high-speed centrifugation and was analyzed by Western blot.

### 2.5. Immunoprecipitation and Western blot

Total protein extracts were separated by SDS-PAGE under reducing conditions. Membranes were incubated with the following primary antibodies: anti-GLUT4 (Santa Cruz), anti-PTP1B, anti-IRS1, anti-IRS2, anti-phosphotyrosine (4G10) (Merk-Millipore), anti-phospho-AKT (Thr308), anti-IR, anti-PTEN, anti-phospho-AKT (Ser473), anti-phospho-p44/42-MAPK (Thr202/Tyr204), anti-AKT (pan), anti-p44/42-MAPK (Cell Signalling). Anti-SV40-LTag and anti-nephrin antibodies were a gift from K. Trygvasson (Stockholm, Sweden). Blots were then probed with mouse monoclonal anti- $\alpha$  tubulin or anti- $\beta$  actin antibodies (Sigma) as protein loading controls. For immunoprecipitation, equal amounts of protein were immunoprecipitated with the corresponding antibodies. The immune complexes were collected on agarose beads and submitted to Western blot analysis.

### 2.6. Cell viability assay

Cell viability was quantified by the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions.

### 2.7. Scratch assay

Cells were seeded to confluence. The scratch was performed using a pipette tip and then, the media was changed. Images of the regions of interest were taken at time 0 and after 18 h. The percentage of cells in the area of the denuded clear zone was measured using the Image J software.

### 2.8. Immunofluorescence

Cells were seeded on glass coverslip slides (Menzel-Gläser, Braunschweig, Germany) and stained with polyclonal anti-GLUT4 antibody (Santa Cruz Biotechnology, Palo Alto, CA). The F-actin cytoskeleton was visualized using phalloidin-conjugated FITC (Invitrogen, Carlsbad, CA). Immunofluorescence was examined in a Nikon Eclipse 90i microscope with imaging software Nis elements (Nikon) or in a confocal microscope LSM710 (Zeiss) with Zen 2011 software (Leica).

### 2.9. Transduction of *Irs2*-deficient podocytes by adenoviral infection

Differentiated mPodIRS2KO were infected with 10 MOI (multiplicity of infection or viral particles per cell) of IRS2 or mock ( $\beta$ -galactosidase) adenoviruses. Podocytes were routinely infected for 36 h as previously described [25], after which mPodIRS2KO recovered IRS2 expression. Then, cells were used for further experiments.

### 2.10. Transient transfection with siRNA

mPodIRS2KO were transfected with 25 nM PTEN siRNA or with a scrambled control siRNA following DharmaFECT General Transfection Protocol. After 48 h, cells were used for experiments. Transfection efficiency was evaluated by real-time qPCR and Western blot.

### 2.11. RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from podocytes using TRI Reagent (Sigma). 1  $\mu$ g of RNA was reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time qPCR was performed with an ABI 7900 sequence detector using the SyBr Green method and d(N)6 random hexamer with the primers for mouse PTEN [26].

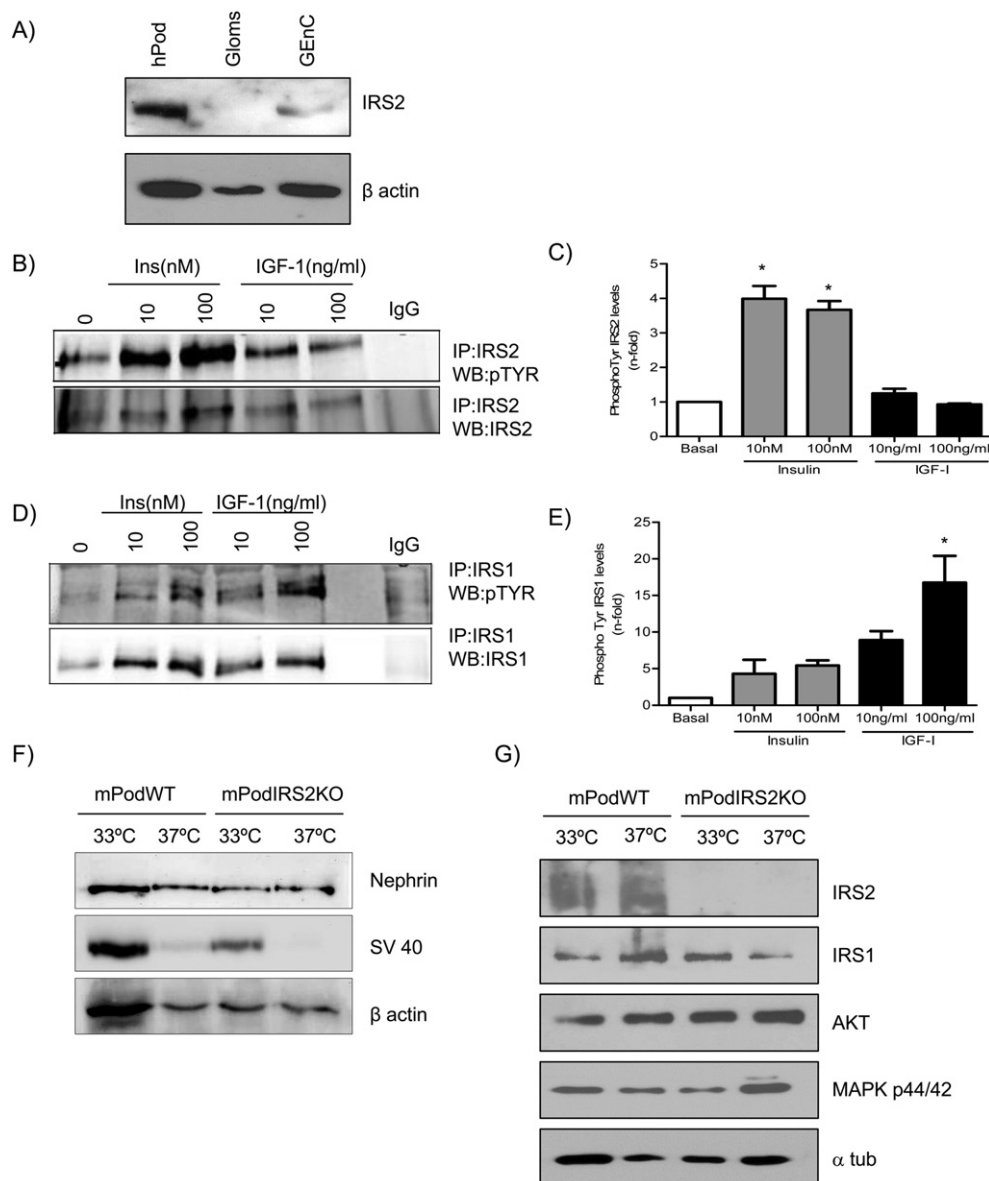
RNA expression was corrected by 18S expression (Fw 5'AGTCCCTGCCCT TGTACACA3' and Rv 5' GCCTCACTAAACCATCCAATCG3').

## 2.12. Short hairpin PTP1B knockdown

Specific shRNA mouse protein tyrosine phosphatase 1B (PTP1B) or control (scrambled) lentiviral transduction particles (Sigma) were used to produce stable knockdown murine podocyte cell lines. These clones were then selected, expanded and examined for their levels of PTP1B. Transfection efficiency was evaluated by real-time qPCR and Western blot.

## 2.13. In vivo studies

Wild-type (*Irs2*<sup>+/+</sup>) and IRS2-deficient (*Irs2*<sup>-/-</sup>) mice were maintained on a similar mixed genetic background (C57BL/6 x 129Sv) as previously described [27]. Anesthesia and perioperative analgesia were used to maintain animal well-being throughout the experiments. Three weeks after the onset of hyperglycemia, urine was collected. All animal husbandry was carried out in accordance with in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Experimental procedures were performed with the appropriate governmental and institutional ethical and legal approval.



**Fig. 1.** IRS2 is expressed in podocytes and is phosphorylated in response to insulin. A) Western blot analysis of IRS2 in a human podocyte cell line (hPod), total glomeruli (Gloms) and in a human glomerular endothelial cell line (GEnC). Loading control of  $\beta$ -actin is shown. B) Conditionally immortalized murine podocytes were stimulated with insulin or IGF-1 at the indicated doses for 15 min. Total protein was immunoprecipitated with anti-IRS2 antibodies and analyzed by Western blot with anti-pTYR or anti-IRS2 antibodies. C) Quantification of IRS2 tyrosine phosphorylation. D) Murine podocytes were stimulated with insulin or IGF-1 for 15 min. Total protein was immunoprecipitated with anti-IRS1 antibodies and analyzed by Western blot with anti-pTYR or anti-IRS1 antibodies. E) Quantification of IRS1 tyrosine phosphorylation. Results are expressed in fold-change over mPodWT controls and are mean  $\pm$  SD. In C) \* $p < 0.01$  vs Basal and in E) \* $p < 0.05$  IGF-1 (100 ng/ml) vs. Basal. F) Western blot analysis of total protein extracts of mPodWT and mPodIRS2KO podocytes at 33 °C and 37 °C. Representative images of nephrin and SV40-LTag are shown. G) Total protein extracts from mPodWT and mPodIRS2KO podocytes at 33 °C and 37 °C were loaded. Representative Western blot of key proteins of the insulin signaling cascade using  $\alpha$ -tubulin as a loading control. Results are representative of 3 independent experiments.



## 2.14. Clinical biochemistry

Urine was collected for 24 h in mouse metabolism cages (Technoplast®). Urinary albumin and creatinine levels were measured using Urinary Albuwell® and Creatinine Companion® kits (Exocell, PA, USA). Blood was collected via cardiac puncture and plasma creatinine levels were measured by HPLC. Creatinine clearance as a measure of estimated glomerular filtration rate (eGFR) was calculated using the following formula: Urine vol (μl) × urine creatinine (mg/dl)/plasma creatinine (mg/dl) × 1440 (min). Results were corrected to total body weight prior sacrifice to provide eGFR.

## 2.15. Transmission electron microscopy

Electron microscopy was performed as previously described [28] with slight modifications. Blinded analysis of glomerular histology was performed by an experienced histopathologist.

## 2.16. Statistical analysis

Results are expressed as mean ± SD of at least three independent experiments. Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL) software. When comparing two groups non-parametric (Mann–Whitney–Wilcoxon and Kruskal–Wallis) analysis was used. When analyzing multiple groups one-ANOVA analysis was performed with bonferroni post hoc and Tukey Kramer multiple comparison test. A p value less than 0.05 were deemed significant.

## 3. Results

### 3.1. IRS2 is enriched in the podocyte within the glomerulus and phosphorylated by insulin

Immortalized human glomerular cell lines demonstrated that IRS2 expression was enriched in the podocyte, with minimal signal detected in either glomerular endothelial cells (GEnC) or whole glomerular lysates (Fig. 1A). Furthermore, wild-type murine podocytes also showed a robust expression of IRS2. Immunoprecipitation studies revealed that insulin rapidly phosphorylated IRS2 in comparison to IRS1 at doses ranging from 10 to 100 nM (Fig. 1B,C). In contrast, IGF-1 stimulation preferentially phosphorylated IRS1 in comparison to IRS2 (Fig. 1D,E).

To further explore the role of IRS2 in the podocyte, conditionally immortalized temperature-sensitive large T antigen *Irs2*<sup>-/-</sup> (mPodIRS2KO) and wild-type (mPodWT) podocyte cell lines were generated from 12-week-old mice. Immortalized podocytes were studied in their differentiated state after 14 days of thermo-switching to 37 °C in which they expressed the podocyte-specific protein nephrin and also the key components of the insulin signaling cascade including IRS1, AKT and p44/42-MAPK similar to the mPodWT controls (Fig. 1F,G). The absence of IRS2 was confirmed in the mPodIRS2KO by genotyping and analyzing protein levels (Supplementary Fig. 1A,1B, Fig. 1G). Importantly, the expression of p27 was up-regulated in both types of podocytes after thermo-switching, illustrating cell cycle arrest and terminal differentiation (Supplementary Fig. 1C).

### 3.2. IRS2 is essential for insulin signaling and insulin actions in podocytes

In response to insulin stimulation, podocytes are able to rapidly absorb glucose via the glucose transporters GLUT4 and GLUT1, and this process is dependent on the filamentous actin (F-actin) cytoskeleton [12] and nephrin [11]. Since IRS proteins act as central integrators of insulin signaling [29], we analyzed the impact of *Irs2* deletion on these parameters. *Irs2* deficiency in podocytes (mPodIRS2KO) resulted in a significant reduction of basal glucose uptake and also in the

complete abrogation of insulin-stimulated glucose uptake (Fig. 2A). *Irs2* loss also reduced insulin-stimulated translocation of GLUT4 from cytosol to the plasma membrane. Additionally, basal level of GLUT4 at the plasma membrane was decreased in mPodIRS2KO consistent with the decreased basal glucose uptake (Fig. 2B, C, D).

*Irs2* deletion also impaired F-actin dynamics and motility in podocytes with a marked reduction of insulin-stimulated cortical F-actin remodeling (Fig. 3A,B) and a reduction of podocyte cell motility measured with scratch assays (Fig. 3C,D).

### 3.3. IRS2 modulates AKT-signaling in podocytes

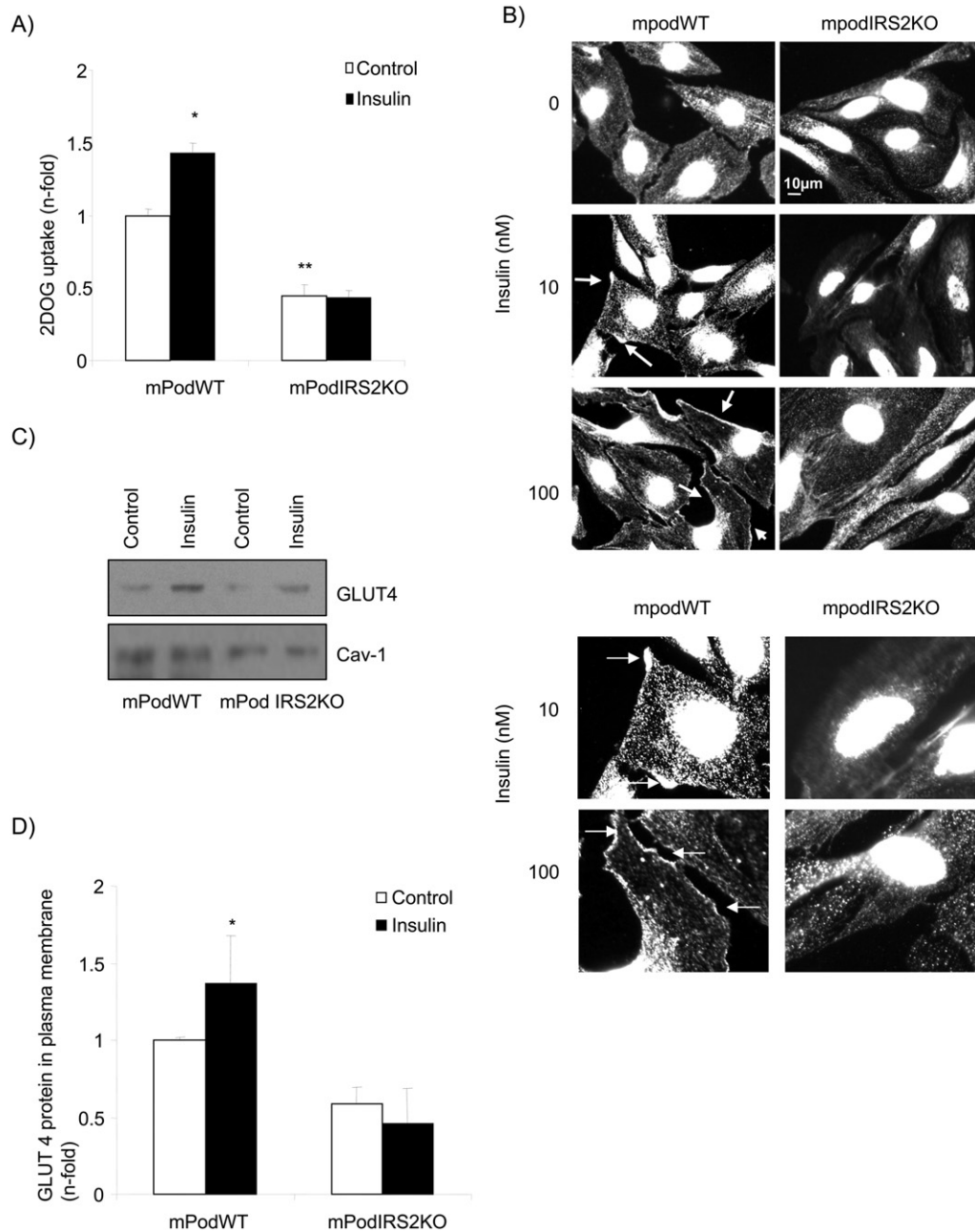
To elucidate the mechanism by which the absence of IRS2 induced insulin resistance in podocytes, we interrogated the insulin-signaling pathway in these cells. Insulin-stimulated insulin receptor (IR) tyrosine phosphorylation was preserved in mPodIRS2KO (Fig. 4A, Supplementary Fig. 2A), as was IRS1 expression and its tyrosine phosphorylation (Fig. 4B, Supplementary Fig. 2B). However, AKT phosphorylation was significantly impaired at both Ser 473 and Thr 308 residues (Fig. 4C,D) when IRS2 was absent. Notably, basal AKT Thr 308 phosphorylation was increased in mPodIRS2KO, but did not further increase in response to insulin stimulation. Insulin stimulated p44/42-MAPK phosphorylation was similar between the two cell types although again, basal phosphorylation levels were higher in mPodIRS2KO. Although we found differences in AKT signaling in mPodIRS2KO and this is critical for insulin action in podocytes [30], we didn't detect differences in the survival properties of mPodIRS2KO in comparison to wild-type podocytes when stressed with puromycin (Fig. 4E).

### 3.4. Knockdown of PTEN, but not PTP1B, rescues insulin sensitivity in *Irs2*-deficient podocytes

We then examined whether we could manipulate the insulin-signaling pathway in *Irs2*-deficient podocytes to rescue insulin sensitivity. We targeted two well-known negative cellular regulators of insulin signaling; protein tyrosine phosphatase 1B (PTP1B) which has been shown to inhibit insulin signaling at the level of IR and is expressed in podocytes [31], and PTEN that regulates AKT activity in the PI3K pathway [32]. No differences were found in PTP1B mRNA (data not shown) or protein expression levels between mPodIRS2KO and mPodWT podocytes (Fig. 5A, B). Furthermore, shRNA-mediated stable PTP1B knockdown in mPodIRS2KO cells that reduced PTP1B mRNA levels by 95% did not rescue insulin-stimulated AKT phosphorylation (Fig. 5C, Supplementary Fig. 2C). Interestingly, we found that the loss of IRS2 caused a prolonged overexpression of PTEN (Fig. 5D, E), and that short-term genetic reconstitution of IRS2 by adenoviral transfer did not restore the insulin response in AKT phosphorylation at Ser 473 or reduce PTEN overexpression (Fig. 5F, G, Supplementary Fig. 2D). However, when PTEN was knocked down using siRNA technology (Fig. 6A, B) it resulted in the rescue of insulin-induced AKT phosphorylation, glucose uptake, GLUT4 translocation (Fig. 6B, C, E) and F-actin remodeling (Fig. 6D, F).

### 3.5. *Irs2*-deficient mice develop early features of diabetic nephropathy

Whole body *Irs2*-deficient mice were assessed for glomerular pathology. We found that male *Irs2*<sup>-/-</sup> mice were markedly diabetic and developed significant albuminuria by 12-weeks of age with a loss of podocyte foot process architecture in comparison to their wild-type (*Irs2*<sup>+/+</sup>) littermate controls (Table 1, Fig. 7, Supplementary Fig. 3A, B). Unfortunately, it was difficult to assess the specific contribution of IRS2 loss to podocyte dysfunction in this setting due to the severity of diabetes. Indeed, age and strain-matched mice rendered equally diabetic by STZ injections also developed



**Fig. 2.** Immortalized podocytes from *Irs2*-deficient mice are insulin resistant in respect to glucose uptake and GLUT4 translocation. A) 2-DOG uptake was measured following stimulation of podocytes with or without insulin. Results are mean  $\pm$  SD of 4 independent experiments and are expressed as fold-change of basal mPodWT. \* $p < 0.05$  and \*\* $p < 0.005$ . B) Immunofluorescence of GLUT4 in mPodWT and mPodIRS2KO podocytes upon insulin stimulation. GLUT4 located at the plasma membrane is indicated by arrows (scale bar 10  $\mu$ m). Note in the magnified sections, that GLUT 4 is unable to translocate to the plasma membrane upon insulin stimulation in mPodIRS2KO. Representative images of 5 independent experiments are shown. C) Representative Western blot detecting GLUT4 in plasma membrane extracts using caveolin 1 as a plasma membrane loading control. D) Quantification of GLUT4 translocation to the plasma membrane analyzed by Western blot. Results are mean  $\pm$  SD of 4 independent experiments. \* $p < 0.05$  vs. basal mPodWT.

similar and significant albuminuric kidney disease (Supplementary Fig. 3C, D).

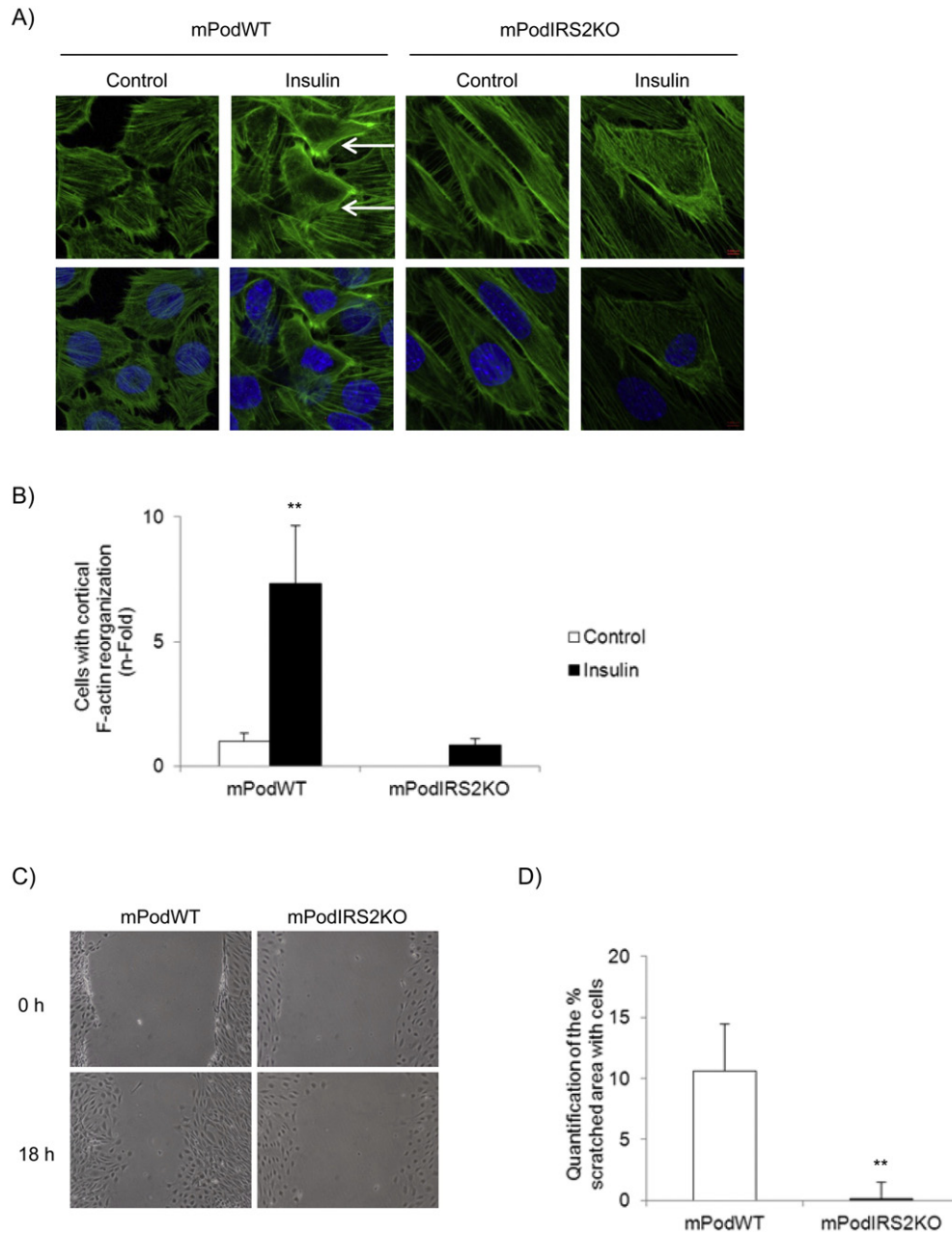
#### 4. Discussion

This work demonstrates that IRS2 is a critical molecule in the insulin signaling pathway in the podocyte. IRS2 loss here results in a severe insulin resistance that is coordinated through the negative regulator of insulin signaling, PTEN.

IRS2 is widely expressed in insulin sensitive tissues and in the brain. Insulin and IGF-1 signaling through IRS2 coordinates life span extension, metabolic regulation and cognition [33]. In the kidney, it has

been shown that IRS2 is expressed in the tubules and increases in diabetes [34]. We now demonstrate that within the glomerulus IRS2 is also enriched in the podocyte compartment which constitutes approximately 10% of total glomerular cells [35].

IRS2 is a key mediator of insulin signaling in podocytes downstream of the IR, as demonstrated by the diminished response of *Irs2*-deficient podocytes to insulin in respect to glucose uptake, GLUT4 translocation, F-actin cytoskeleton organization and cell motility. At the molecular level, *Irs2* deficiency here resulted in an impaired insulin-mediated AKT phosphorylation despite intact IR and IRS1 tyrosine phosphorylation responses. These results are consistent with previous data from our laboratory demonstrating the inability of IRS1 to activate PI3K in

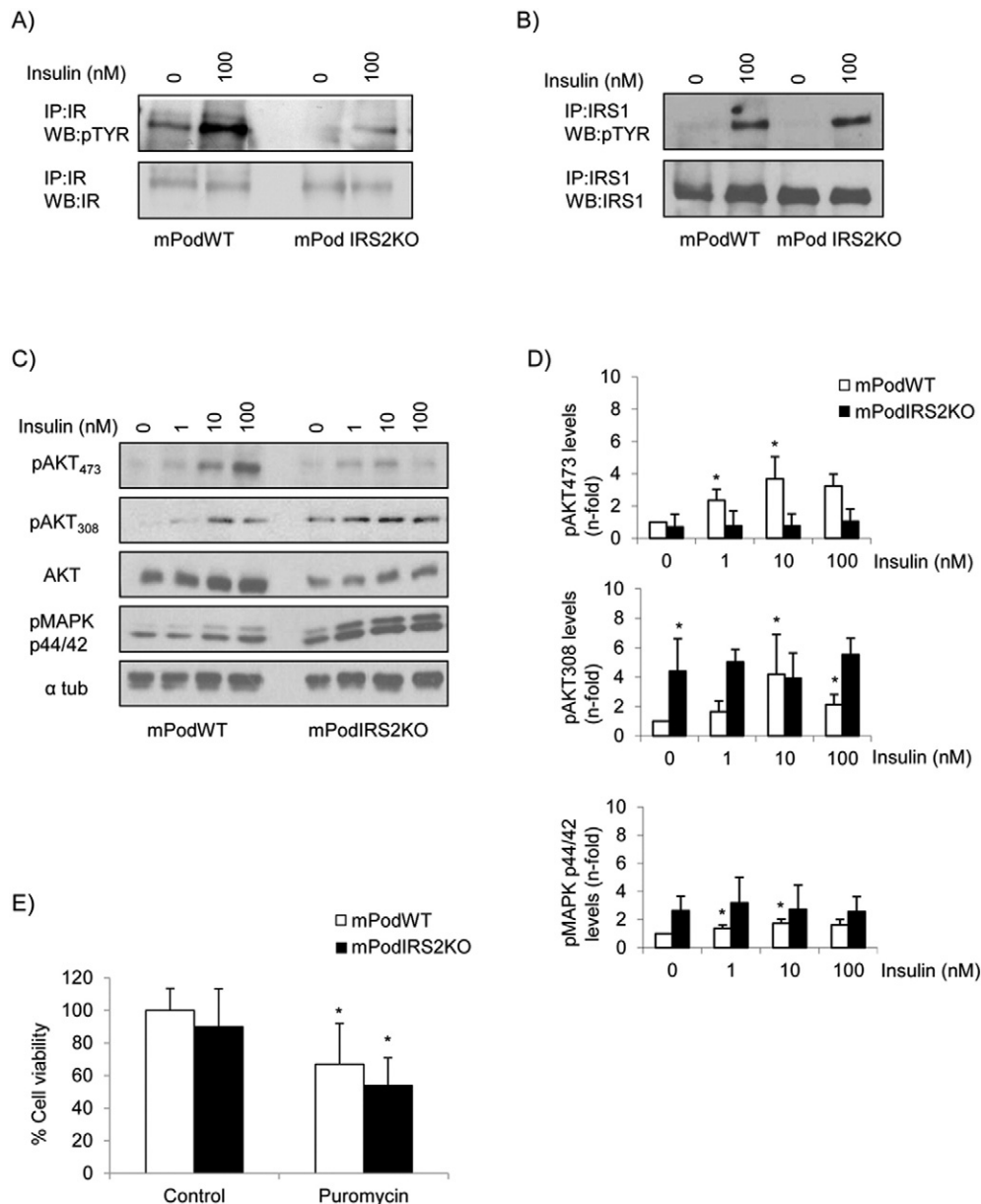


**Fig. 3.** Irs2-deficient podocytes have impaired insulin-mediated F-actin cytoskeleton rearrangement and motility. A) F-actin cytoskeleton was analyzed by immunofluorescence with phalloidin staining. Insulin-stimulated mPodWT cells show cortically reorganized F-actin (arrow), but cortical reorganization was not observed in mPodIRS2KO cells (scale bars 5000  $\mu$ m). Representative images are shown. B) Quantification of cell number with F-actin cortical reorganization using Image J software. Results are mean  $\pm$  SD of 4 independent experiments. \*\*p < 0.001 vs. other groups. C) Representative images of the wound healing at 18 h after scratching. Original magnification 100 $\times$ . D) Quantification of the percentage of cells in the scratched area. Results are mean  $\pm$  SD of 5 independent experiments. \*\*p < 0.001 vs. mPodWT.

response to insulin or IGF-1 in the liver and retina of *Irs2*<sup>-/-</sup> mice [25, 27,36], and also with data in podocytes from *db/db* mice that show a blockade in insulin-induced AKT phosphorylation despite preserved IRS1 levels [13]. This is relevant as both IRS1 and IRS2 can efficiently activate the PI3K and MAPK pathways in other insulin sensitive tissues [33] and highlights the cellular specificity of IRS2 as a critical node of the insulin signaling pathway in podocytes. Conversely, elsewhere in the glomerular filtration barrier, in glomerular endothelial cells, IRS1 has been shown to be an essential mediator of insulin signaling, indicating a high degree of cellular specificity of IRS protein functions within glomeruli [37]. Based on that, our data suggest distinct functions of IRS1 and IRS2 in the podocyte in regard to insulin signaling (through IRS2) and IGF-1 signaling (through the IGF-1R). It is also interesting that there was no obvious compensation for IRS2 and insulin sensitivity

loss through IRS1. Therefore, specific effects of insulin [15] and IGF-1 [38] in podocytes might be the result of the differential activation of IRS2 or IRS1, respectively. Elucidating the cellular signaling pathways for these two hormonal axes will allow specific targeting and manipulation of the insulin and IGF systems.

The involvement of IRS proteins in insulin-mediated AKT signaling, GLUT4 translocation or glucose uptake differs depending on the cellular context. In L6 myotubes, IRS1 activation increases insulin-induced glucose uptake, GLUT4 translocation and actin remodeling whereas *Irs2* deletion does not affect these responses [39]. However, in brown adipocytes, insulin-mediated glucose transport and GLUT4 translocation are through IRS2, suggesting that podocytes and adipocytes display similar requirements of IRS2 in respect to insulin stimulated activation of AKT, GLUT4 translocation and glucose uptake [40]. Interestingly, it



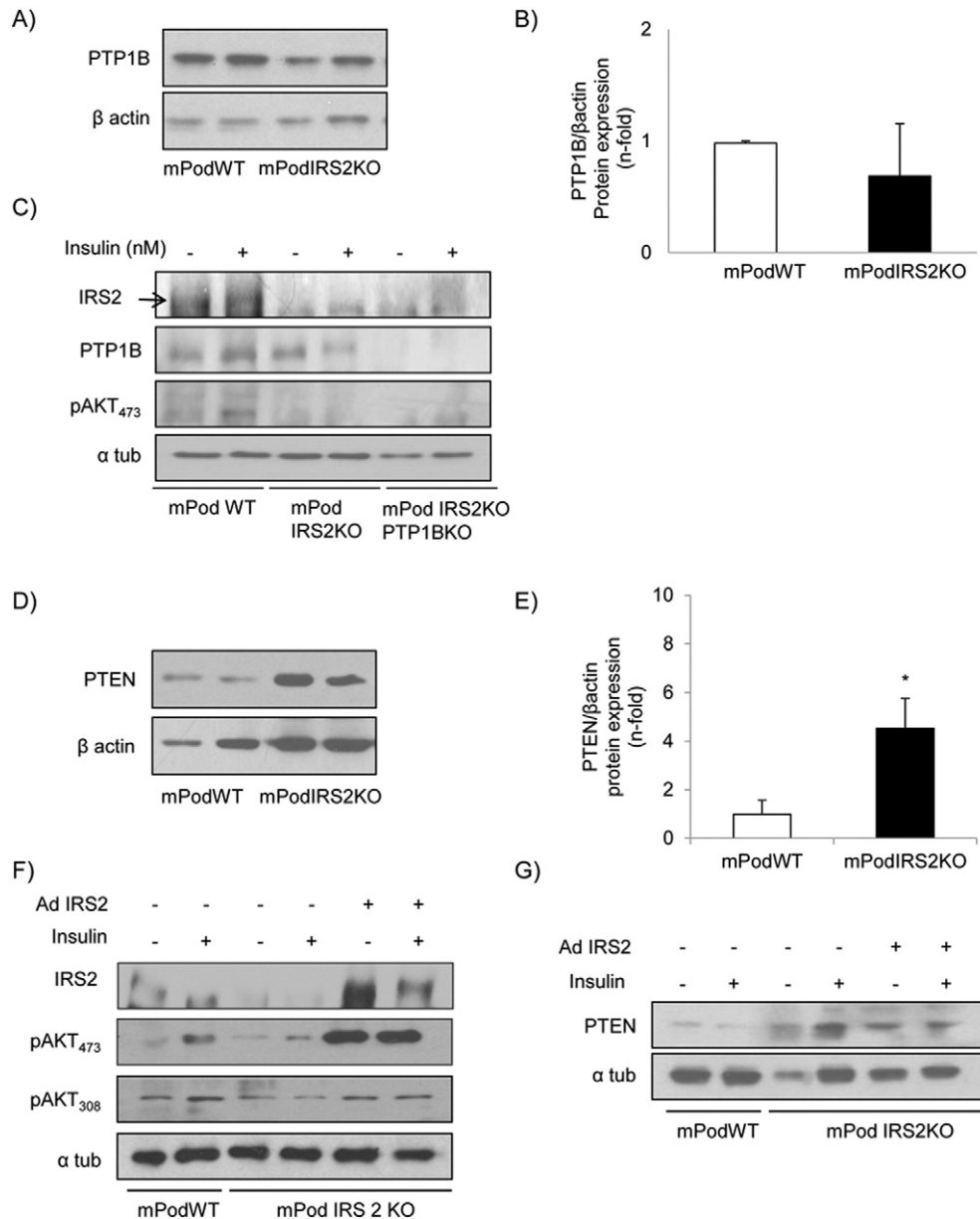
**Fig. 4.** Impaired AKT signaling in Irs2-deficient podocytes. Podocytes were serum-starved and stimulated with insulin. Total protein was immunoprecipitated with A) anti-IR or B) anti-IRS1 antibodies and analyzed by Western blot with anti-pTYR and anti-IR or anti-IRS1 antibodies. Results are representative of 3 independent experiments C) Representative Western blots using the indicated antibodies. D) Quantification of Western blot analysis. Results are mean  $\pm$  SD of 5 independent experiments. \*p < 0.05 vs. basal mPodWT. E) Podocyte cell viability post-puromycin stress. mPodIRS2KO and mPodWT were serum-starved and stimulated with puromycin. Results are mean  $\pm$  SD of 5 independent experiments. \*p < 0.05 vs. their respective controls.

has recently been shown that there are insulin-independent roles of GLUT4 in podocytes that modulate their size and number [41], although it is unknown if these effects are modified by the IRS family. It is also clear that AKT2 has an important role in podocytes viability [30]. However, we didn't detect differences in the susceptibility to cell death of mPodIRS2KO indicating the existence of additional mechanisms independent of IRS2 that modulate podocytes viability.

After discovering that the loss of IRS2 rendered the podocytes insulin resistant, we explored the mechanism underlying this effect and attempted to reverse it. We focused on PTP1B [42] and PTEN [43], two relevant molecules characterized as negative modulators of insulin signaling. Our previous data have demonstrated that the suppression of PTP1B in the liver of *Irs2*<sup>-/-</sup> mice can restore insulin signaling through an IRS1-mediated PI3K/AKT signaling pathway [27]. This was not the case in the podocyte. This, again, demonstrates the cellular specificity of insulin signaling pathways in different tissues, but is not entirely

surprising as the negative effects on insulin actions of PTP1B are predominantly mediated through the IR and IRS proteins [42], which are proximally located in the insulin signaling cascade. We then targeted PTEN, a lipid phosphatase that dephosphorylates PtdIns-3,4,5-P3 *in vivo* and is a negative modulator of insulin signaling downstream of the IR/IRS complex [44–46]. Recently, it has been shown that the impairment of insulin induction of glucose uptake into podocytes cultured in the presence of high glucose concentrations for long time-periods is associated with increased PTEN levels [32]. We found that *Irs2* deficiency increased PTEN levels which elicited a negative regulation of AKT by dephosphorylating the Ser 473 residue as described [47]. Moreover, this constitutive PTEN elevation might further impact the function of other phosphatases since we found up-regulated basal Akt Thr 308 phosphorylation. This effect seems to be the consequence of the cellular adaptation to prolonged *Irs2* deficiency, since acute (36 h) IRS2 reconstitution into podocytes failed to restore PTEN back to normal levels





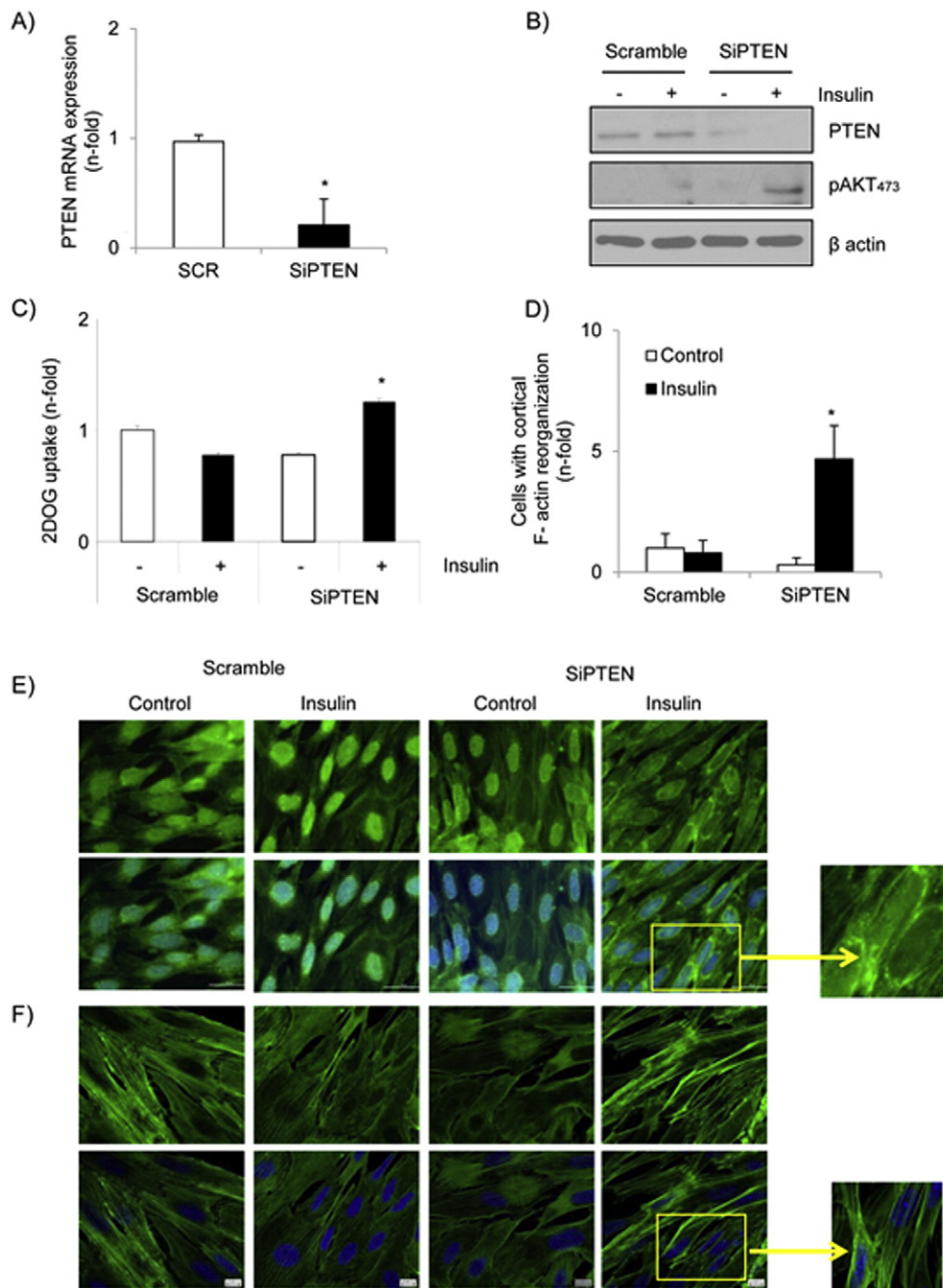
**Fig. 5.** Differential expression of negative modulators (PTP1B and PTEN) of insulin signaling in podocytes from wild-type and *Irs2*-deficient mice. A) Representative Western blot with anti-PTP1B antibody B) Quantification of PTP1B protein expression. Results are mean  $\pm$  SD of 4 independent experiments. C) Podocyte cell lines (mPodWT, mPodIRS2KO, mPodIRS2KO/PTP1BKO) were serum-starved and stimulated with insulin. Western blot analysis of IRS2, PTP1B, pAKT (Ser 473) and  $\alpha$ -tubulin as a loading control. Similar results were obtained in 3 independent experiments. D) Western blot analysis of PTEN in non-stimulated podocytes. E) Quantification of PTEN protein expression in non-stimulated podocytes. Results are mean  $\pm$  SD of 4 independent experiments. \* $p < 0.05$  vs. mPodWT. F) mPodIRS2KO were infected with adenoviruses encoding IRS2 (Ad IRS2) or GFP as a mock control. After 36 h, cells were serum-starved and stimulated with insulin. Western blot analysis with the indicated antibodies. Five different experiments were performed with similar results. G) Western blot analysis with anti-PTEN antibody using  $\alpha$  tubulin as a loading control. Similar results were obtained in 3 independent experiments.

and did not reverse insulin resistance. Encouragingly, PTEN silencing rescued insulin-induced AKT signaling in *Irs2*-deficient podocytes and, as a consequence, also rescued insulin-induced GLUT4 translocation, glucose uptake and F-actin cytoskeleton reorganization. These results suggest that PTEN constitutes a key point of regulation of insulin signaling in *Irs2*-deficient insulin resistant podocytes. Our findings are in agreement with data of Kushner and co-workers that found increased AKT and Foxo1 phosphorylation in the islets of *Irs2*<sup>-/-</sup>/*Pten*<sup>+/-</sup> mice [48].

A reciprocal interaction between IRS2 and PTEN has been described in tumor cell lines. In MDA-MB-468 breast cancer cells, reduced PtdIns-3,4,5-P3 levels elicited a feedback mechanism involving IRS2 up-regulation that attempted to restore PtdIns-3,4,5-P3 content in order to circumvent apoptosis and growth inhibition [49,50]. Conversely,

PTEN knock-down in mouse embryonic fibroblasts decreased IRS2 expression [49,50]. We show an inverse reciprocal modulation in which *Irs2* deletion increases PTEN, thereby blocking AKT phosphorylation downstream of IRS proteins. Thus, targeting PTEN in *Irs2*-deficient podocytes can sensitize this cell to insulin which may be beneficial in preventing kidney disease, particularly in DN. A recent study in podocytes found that PTEN inhibition altered cytoskeletal dynamics, resulting in changes in the permeability and barrier function [51]. Differences in the podocyte origin could account for such differences. It should also be remembered that PTEN is a tumor suppressor and great care should be taken in therapeutically targeting PTEN in insulin resistance, especially if it has systemic effects on other cell types.

Finally, we extended our study to examine the glomerular effects of IRS2 in whole body *Irs2*-deficient mice. These mice developed severe



**Fig. 6.** PTEN silencing rescues insulin-mediated AKT phosphorylation, glucose uptake, GLUT4 translocation to the plasma membrane and F-actin cytoskeleton rearrangement in *Irs2*-deficient podocytes. mPodIRS2KO podocytes were transfected with control (scrambled-SCR) or PTEN siRNAs for 48 h followed by stimulation with insulin. A) *Pten* mRNA quantification using *18S* as a housekeeping gene. Results are mean  $\pm$  SD of 4 independent experiments. \* $p < 0.05$  vs. SCR. B) Protein extracts were analyzed by Western blot with the indicated antibodies. Three independent experiments were performed with similar results. C) 2-DOG uptake was measured following stimulation with or without insulin. Results are expressed as fold-change versus mPodIRS2KO podocytes transfected with control scrambled siRNA. Results are mean  $\pm$  SD of 4 independent experiments. \* $p < 0.05$  vs. mPodIRS2KO siPTEN. D) Quantification of number of cells with F-actin cortical reorganization using Image J software. Results are mean  $\pm$  SD of 4 independent experiments. \* $p < 0.01$  vs. other groups. E) GLUT4 translocation to the plasma membrane was analyzed by immunofluorescence. Localization of GLUT4 at plasma membrane is indicated by an arrow (scale bars 100  $\mu$ m). A magnification area indicates GLUT4 translocation to the membrane. Representative images are shown. F) F-actin cytoskeleton reorganization was analyzed with phalloidin staining (scale bars 10,000  $\mu$ m). Insulin-stimulated F-actin reorganization is indicated by an arrow. Representative images are shown.

diabetes and kidney disease, but we are unable to decipher if this was partially or completely due to the loss of IRS2 in the podocyte or to the systemic effects of diabetes. Going forward it will be interesting to specifically delete IRS2 from the podocyte to explore this issue more fully in non-diabetic and diabetic environments. Based on these and previous findings, we conclude that IRS2 has differential functions in different parts of the kidney. In the tubules it modulates renal fibrosis during DN [34,52], and in the podocyte it is important for insulin

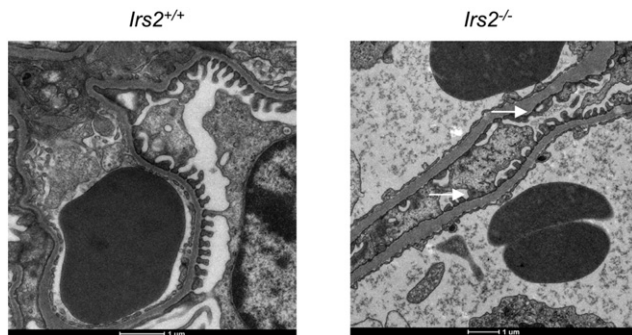
sensitivity, glucose uptake and cytoskeleton function. Similarly, differing roles of mTORC1 between the glomeruli and tubules in DN have also been reported [53,54].

## 5. Conclusion

As summarized in Fig. 8, this work reveals that IRS2 is a key molecule in insulin signaling in the podocyte since its deficiency causes insulin

**Table 1**Renal clinical-pathological correlates in male wild-type (*Irs2*<sup>+/+</sup>) and *Irs2*<sup>-/-</sup> mice.

| Mean Age                                    | 5–6 weeks                  |                            | 13–14 weeks                |                            |
|---|----------------------------|----------------------------|----------------------------|----------------------------|
|   | <i>Irs2</i> <sup>+/+</sup> | <i>Irs2</i> <sup>-/-</sup> | <i>Irs2</i> <sup>+/+</sup> | <i>Irs2</i> <sup>-/-</sup> |
| Fasting Blood Glucose (mmol/l)              | 7.25 ± 0.23 (8)            | 12.6 ± 1.06 (8)***         | 7.36 ± 0.389 (10)          | 25.16 ± 3.39 (6)***        |
| Urine volume (ml/day)                       | 0.70 ± 0.13 (8)            | 0.95 ± 0.21 (6)            | 0.54 ± 0.09 (10)           | 33.94 ± 4.93 (6)***        |
| UAE (μg/day)                                | 11.23 ± 2.08 (8)           | 7.52 ± 1.87 (6)            | 8.60 ± 2.25 (10)           | 77.48 ± 24.11 (6)***       |
| UACR (μg/mg)                                | 29.16 ± 3.18 (8)           | 33.10 ± 6.08 (6)           | 27.53 ± 4.42 (10)          | 72.37 ± 16.46 (7)**        |
| Serum creatinine (μmol/l)                   | 8.84 ± 1.76 (5)            | 9.72 ± 0.88 (5)            | 12.38 ± 0.88 (9)           | 11.49 ± 0.88 (7)           |
| Creatinine clearance (μl/min/g body weight) | 12.40 ± 1.26 (5)           | 10.16 ± 3.20 (5)           | 4.76 ± 1.10 (9)            | 36.43 ± 5.66 (6)***        |

**Fig. 7.** Diabetic *Irs2*<sup>-/-</sup> mice have podocyte foot process effacement. A) Representative TEM images of kidneys from wild-type and *Irs2*<sup>-/-</sup> mice. Four mice per group were analyzed. Foot process widening in kidneys from *Irs2*<sup>-/-</sup> mice is shown (white arrows).

resistance in podocytes through up-regulation of PTEN. Importantly, suppressing PTEN rescues insulin sensitivity in podocytes and restores their functions.

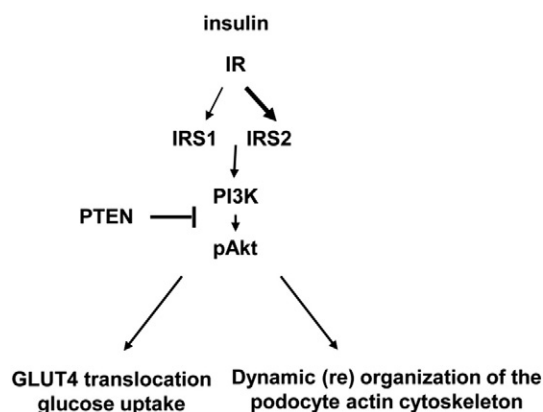
Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2015.09.020>.

### Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript. RJC is in receipt of an academic grant from Novo Nordisk.

### Contributions

BS, RJC, AMV conceived and/or designed the work that led to the submission. BS, EM, AL, RMC, GIW, LN, LJH, DPB, AMV acquired data

**Fig. 8.** Schematic representation of the mechanism by which *Irs2* deficiency promotes insulin resistance leading to impaired glucose uptake and dynamic reorganization of podocyte F-actin cytoskeleton.

and/or played an important role in interpreting the results. BS, RJC, AMV drafted the manuscript. BS, AGR, AO, MAS, DPB, RJC, AMV revised the manuscript. All authors approved the final version.

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